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By

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ABSTRACT

Lyme disease is a pervasive illness caused by the transmission of the spirochete *Borrelia burgdorferi* from the bite of an infected black-legged tick, *Ixodes scapularis*. Ticks initially obtain the spirochete by feeding on an infected animal host. Ticks feed on a broad range of hosts, but some of these hosts are more competent carriers of Lyme disease and more readily transmit *B. burgdorferi* to feeding ticks. Thus, knowing what host a tick has fed on could provide valuable information in studying the transmission of Lyme disease. However, studying the relationships between ticks and their hosts has proved to be a challenging task.

One possible option for determining a tick's host could be found in Stable Isotope Analysis (SIA), a technique often used for food web studies that provides unique signatures based on the concentration of stable isotopes found in the analyzed subject. The animal being tested and the diet they consumed, largely determines these signatures.

I build on previous SIA work by testing how ticks isotopic signatures vary when Eastern chipmunk hosts, Tamias striatus, are fed different diets. Past work in our lab suggests that incorporation of carbon stable isotope signatures is quick and sensitive. This leads to concern that individual diets may significantly distort the isotopic signatures of carbon in tick hosts and may not be a valuable indicator.

Chipmunks were captured in Reist Sanctuary and held in the animal care facility. Upon their arrival nymphal ticks were placed on the chipmunks to feed.

Meanwhile the chipmunks were fed a variety of regimented diets (corn, insect, sunflower seed, and meat). Ticks that engorged and dropped were collected and animals were released. Adult ticks were processed and submitted for SIA along with samples of each diet the chipmunks were fed for comparison.

The results from the stable isotope analysis showed no significant variation among diet groups. The signatures from ticks did not match the corresponding host diets. These results imply that nitrogen signatures are not as readily incorporated in ticks as carbon is, suggesting that nitrogen could provide valuable insight in regards to understanding tick-host interactions without being sensitive to sudden dietary changes.

TABLE OF CONTENTS Page

Introduction:

Over the past few decades Lyme disease has become a prominent problem in the United States. Despite increased awareness and study, the tick-borne illness still infects as many as 300,000 Americans every year. Many people who contract Lyme disease suffer from its symptoms of fatigue, fever, aching, swollen joints, chills, and headaches (CDC, 2016). These common symptoms often lead to misdiagnosed cases of Lyme disease that go untreated despite the disease's prevalence. This heightens the severity of the disease and leads to more harmful symptoms, characteristic of the later stages of the disease. These symptoms include, but are not limited to, arthritis, facial palsy, nerve pain, short-term memory issues, and heart palpitations (CDC, 2016). Since Lyme disease is so often misdiagnosed it is best take preventative measures. However, this can only be accomplished by studying the ecology of the disease.

Lyme disease arises from the bite of a black-legged tick, *Ixodes scapularis,* which act as a vector for the disease-causing spirochete bacterium, *Borrelia burgdorferi.* How exactly this bacteria is acquired and transmitted is closely tied to the unique physiology and life cycle of the black-legged tick. The tick lifecycle is dependent on a complex system of feeding on multiple hosts during different life stages. Every time a tick feeds it has a chance of acquiring *Borrelia burgdorferi* from an infected host, or spreading it to an uninfected host. However, because ticks feed off numerous hosts in a lifetime, it is difficult to identify a tick's last host. Ticks have three life phases, starting as larva, then progressing to nymphal and adult ticks with

each feeding. To begin, larval ticks must find a host by questing and feeding before falling off and molting into nymphs, which remain dormant until they look for another host to feed on. At this stage the ticks will again fall off and molt into their adult stage. As adults, ticks will feed on another host while looking for a mate residing on the same host. If mating is successful, engorged females will fall off of the shared host in order to lay hundreds or even thousands of eggs (Ostfeld, 2013). It is documented that mated females will often enter a rapid feeding phase, or "big sip," in which they suck out a host's blood at a relatively fast rate to engorge themselves with large quantities of blood in the final 12 to 36 hours of feeding (Sonenshine, 2014). It is probable that ticks of all genders and at other life stages may implement a similar rapid feeding phase in their own engorgement process.

The importance of identifying a tick's host is made clear through the concept of host "reservoir competence" or the propensity of a host to contract, replicate and spread the Lyme disease spirochete. Different species of animals have a wide range of reservoir competence, for example, eastern chipmunks have a much higher reservoir competence than white tailed deer and thus, have a much higher chance of spreading Lyme disease (LoGiudice et al., 2003). Knowing what host species a tick previously fed on, along with the knowledge of the reservoir competence of that species, provides valuable insight on whether or not the tick is likely to be infected with *B. burgdorferi* and whether some other species have been underappreciated as tick hosts due to the shortcomings of methods used so far.

Many different methods have attempted to track and better understand tick feeding habits on various hosts. For example, DNA analysis approaches such as

reverse line-blot hybridization, heteroduplex analysis, PCR-restriction fragment length polymorphism and multiplexed PCR have been used to study bloodmeal samples for various insects that rely on blood from their hosts for nourishment (Gómez-Díaz and Figuerola, 2010). These methods attempt to identify the host a tick has fed on by isolating host DNA in the bloodmeal (Gómez-Díaz and Figuerola, 2010). However, since ticks are typically collected for sampling when they are questing, months after the acquisition of the bloodmeal, the host DNA has often degraded significantly by the time the sample is tested. These methods are successfully used in species such as mosquitos, captured soon after the acquisition of blood meal, but have been unreliable in ticks (Gómez-Díaz and Figuerola, 2010, Hamer, 2015). The lack of reliable methods for testing tick feeding relationships led to a search for other, more dependable techniques.

One of these techniques is known as stable isotope analysis or SIA. Stable isotopes are naturally occurring variations of the abundant elements that are found in all matter, including living organisms. The distinction between a stable isotope and a regular element can be attributed to its extra neutron, which results in a more massive element that does not decay into another element. This heavier mass allows stable isotopes to be isolated and distinguished from their more common elemental partners using a thermal ionization mass spectrometer. The distinguishing of stable isotopes from more abundant elements allows the establishment of a ratio of concentrations between the heavier isotope and its more prevalent form, measured in parts per thousand or "per mil" (‰, Ben-David and Flaherty, 2012). These values are calculated using the sample values compared to standard values: $deltaX =$

$\left[\left(\frac{R_{SAMPLE}}{R_{STANDARD}} - 1\right)\right] * 100$ (Fry, 2006). A delta (δ) value is often unique, due to many natural and physical variables. When δ values from multiple elements are compared, an "isotopic signature" is established. Different biological processes influence signatures in different ways. This is largely due to the variation in the masses of elements and their isotopes which causes the two to behave differently in biological and physical processes. This phenomenon is known as fractionation (Fry, 2006). The isotopic signature of a diet is reflected in the tissues and bodily fluids of a consumer. SIA can use these signatures to discern information about the diet or lifestyle of a subject through the biological or physical implications of the isotopic concentrations.

SIA has many applications in the field of ecology. Various ecological studies have utilized SIA, revealing trophic interactions that would otherwise be difficult to study. For example, C_3 and C_4 plants produce distinct isotopic signatures (Kelly, 2000). C_4 plants have much higher concentrations of ^{13}C when compared to C_3 plants. This is largely due to the differences between the photosynthetic pathways of C_3 plants (such as wheat), which typically have low ^{13}C values, and C_4 plants (such as corn) that typically have enriched ^{13}C values. This allows for accurate identification of C_3 and C_4 plants in the diets of consumers and is one of the main uses of carbon isotope signatures. Corn has a distinct enriched ^{13}C signature and is ubiquitous in food products in the United States, making it a great isotopic marker. Stable isotopes of nitrogen are also used in food web studies. Larger delta values of ^{15}N isotope are indicative of the consumption of animal products. A phenomenon, called trophic enrichment, causes nitrogen isotopes to react to different trophic

levels by changing in a predictable way, increasing in ^{15}N between 2‰ and 4‰ for every trophic level. Various ecological studies have implemented these findings to reveal dietary relationships. For example, Baudin (2013) conducted a study on two species of mice that occupied the same niche. The study used SIA to accurately understand the diet of both species and confirm whether they did in fact share the same food sources. SIA signatures of both species' ^{13}C and ^{15}N showed close similarities, implying that both did in fact share the same diets. Tests like these are extremely helpful in revealing aspects of ecology that would otherwise be very difficult to explore. The applications of SIA in the field of ecology have a wide range that is only limited by the creativity of its user.

One of these applications is the use of SIA in studying tick-host relationships. Recent experiments touch upon and test the idea that the isotopic signature of a tick may vary depending on the host it feeds on. Hamer et al. (2015), conducted a study with lone star ticks, *Amblyomma americanum* that fed on chickens compared to blood samples of a variety of species. The isotopic signatures of the ticks (even at different ages) closely resembled one another in despite the significant variation of the other samples. Thus, they suggested that it might be possible to identify a tick host through isotopic signatures of the tick. Schmidt et al. (2011) conducted a proof of concept study that showed that the expected trophic enrichment between host and tick occurred.

The diet of the host will also influence the isotopic signature of the host and therefore, the tick as well. Belivanov (2015) conducted a study that observed how changing the diets of mealworms (corn, wheat and flour) fed to spiders, caused

predictable changes in spider isotopic signatures, specifically in regards to corn. Spiders that fed on corn-fed mealworms had significantly enriched $\delta^{13}C$ when compared to spiders that fed on wheat or flour. LoGiudice et al. (2017), building on this information, utilized corn as a dietary tracer in mice and chipmunks. The ticks that fed on corn-fed animals were enriched in $\delta^{13}C$ despite a very short feeding period (96 hours). The findings of this study suggest that the isotopic signature of a host (and thus that of a feeding tick) might vary with the short-term diet of the host. Since many wild animals are opportunistic feeders that utilize atypical foods when available, this could be a problem for host identification. Additionally, the study showed no variation in signatures between mice and chipmunks that were fed the same diets, implying that there were no species-specific metabolic differences in fractionation of these isotopes.

My study will help address many questions regarding the identification of tick hosts through the SIA of ticks themselves. Due to the myriad variables to consider with SIA, I will primarily focus on the most prominent ones: how the timing of a diet impacts isotopic signals in a black-legged tick as well as how various host diets impact tick isotopic signatures.

Preliminary studies must be done to better understand how isotopic signatures react to different host diets, as well as how the timing of tick feeding reflects dietary changes in the host. I explored these concepts through one experiment focusing on chipmunks, *Tamias straitus,* and black-legged ticks *I. scapularis*. The experiment involved feeding chipmunks a consistent diet with unique isotopic properties, such as corn, insects and meat over a four-day period.

Ticks were then collected and organized over the four-day period in order to provide insight on the time it took ticks to engorge and fall off, relative to the diet of the chipmunk host.

My experiment addressed the question of whether or not the isotopic signature of a tick would vary with the host's diet. Since corn had already been established as a clear and potent dietary indicator for carbon, I focused on nitrogen isotopes and how sensitive their signatures were in a given timeframe. Based on previous findings, and the nature of isotopic signatures, I predicted that a tick's isotopic signature would change in a way that resembles the isotopic signature of the host's diet.

Methods:

All animals were captured in Reist Sanctuary, an 111-acre forest predominantly populated with white pine, pitch pine, hemlock, red, white and black oak, and maple trees, as well as observed high chipmunk populations. Traps were prepared in locations judged likely to capture chipmunks. Two traps were placed at each site and were baited with oats and sunflower seeds in the afternoon, and checked the following day at four-hour intervals. Chipmunks or mice found in traps were assessed based on their condition, gender and reproductive status. Animals that were lactating, pregnant, or recaptured were released, the rest were taken back to the Union College animal care facility.

Animals were restrained in a wire cone to prevent grooming and 30 larval and 5 to 9 nymphal black-legged ticks were transferred to each animal, which were

left in the cone for twenty minutes to allow the infested ticks to imbed (Sonenshine, 2004). Once the infestation period had concluded, the animals were released into individual cages. The animals were contained in wire mesh cages that were suspended in tubs. The animals were held for four days, allowing the infested ticks enough time to engorge themselves and subsequently fall off into the tub below for collection.

Tubs were thoroughly searched for engorged ticks each morning and the chipmunks were closely monitored. The collected ticks were placed in vials lined with moistened plaster of Paris. Ticks that dropped off in the first 12 to 24 hours were determined to be "wild-diet," as they had fallen off before any significant dietary manipulations could be made.

Every morning and evening, the animals were fed diets that mimicked the isotopic signatures of granivorous, carnivorous and insectivorous diets. I attempted to establish two dietary regimes: a constant and a "pulse" diet. In the former, corn, insects, or meat were provided for the entire four-day captive period, while the second regime involved switching the animals from a neutral diet of sunflower seeds to a special diet for a single day. However, due to trouble feeding the chipmunks, most diets were realized as a one to two day pulse of a specialized diet following a neutral or wild diet. To control for individual animal dietary preferences, chow was prepared to contain a homogenized concentration of corn, insects and meat. However, animals refused to eat the chows and therefore, the diets were manipulated to include corn, insects and pasture fed beef mixed with peanut butter

to enhance palatability. Consumption was closely observed and recorded by noting the quantity of neutral-deviant foods consumed.

After four days, the animals were released at their point of capture. The engorged nymphal ticks were monitored until they molted into adults. The molted adults (n=34) were then washed, killed by freezing, dried in a drying oven at 60°C and prepared for the mass spec, along with samples of the foods consumed by the chipmunks.

Results:

The mean nitrogen and carbon isotopic signatures of ticks were compared using 1-way ANOVAs, with host diet as the independent variable. All statistical

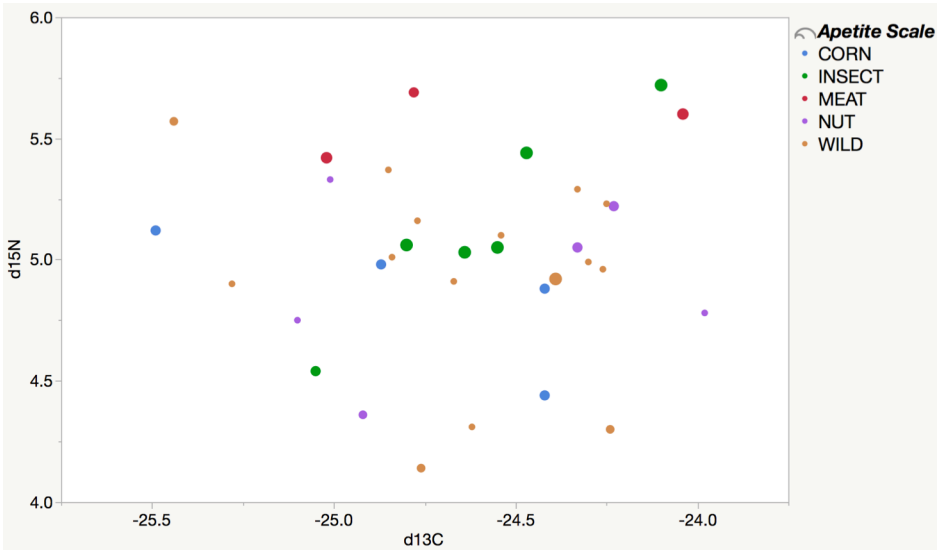

Figure 1. The δ^{15} Nitrogen and δ^{13} Carbon isotope signatures of ticks processed via Stable Isotope Analysis (n=34). The colors of the points correspond to host diet as shown. The size of each data point denote how much of the corresponding diet was consumed by the chipmunk host.

analyses were conducted in JMP. There was no obvious pattern in δ^{15}N or δ^{13}C for ticks that were fed on hosts consuming different diets (Figure 1.). There were no

significant differences in average $\delta^{13}C$ or $\delta^{15}N$ among any of the dietary groups of ticks ($p=0.94$ and $p=0.089$, respectively; Figure 2).

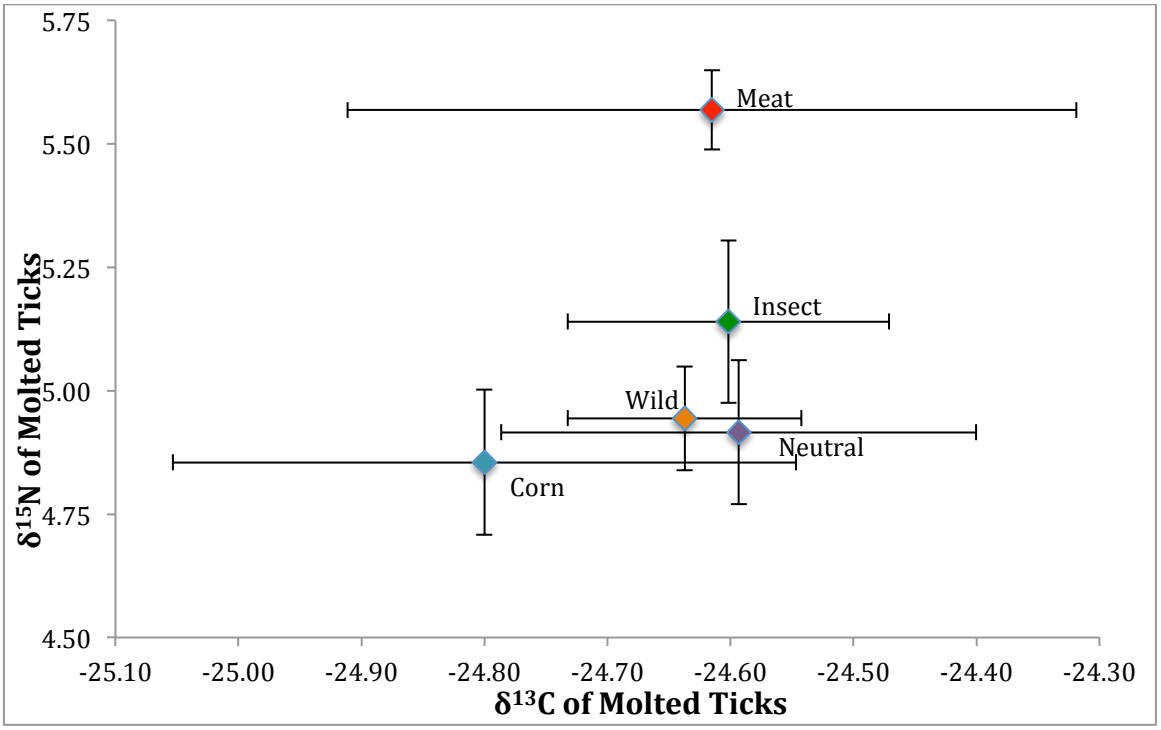

Figure 2. Mean isotopic signatures of each dietary group of ticks: meat (n=3), insect (n=6), wild (n=15), Neutral (n=6), corn (n=4). Error bars denote the standard error for each group.

These data showed a lack of significant difference between each diet group. This unexpected lack of significant variation was further investigated through the isotopic signatures of each diet that the chipmunks fed on (Figure 3.) to see if the pattern of signatures in the ticks resembled those of the diets.

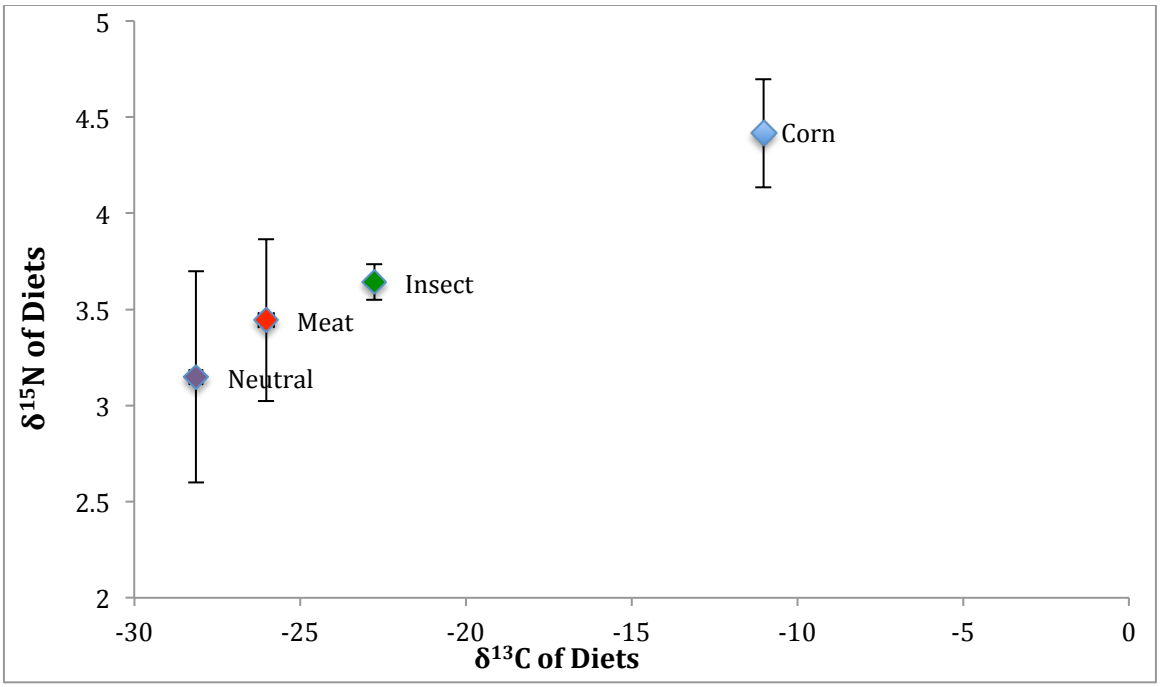

Figure 3. Means of the isotopic signatures of each analyzed diet (n=3). Error bars denote the standard error of each group. Note that standard errors for the X-axis are obscured by the points.

The delta value ranges for each food group differed from the ticks, generally having lower $\delta^{15}N$ signatures with a wider range than the tick values, and a much wider range for $\delta^{13}C$ signatures (Figure 2.). When food samples and ticks $\delta^{15}N$ signatures were compared, the expected trophic enrichment from food to tick was not seen (Figure 4).

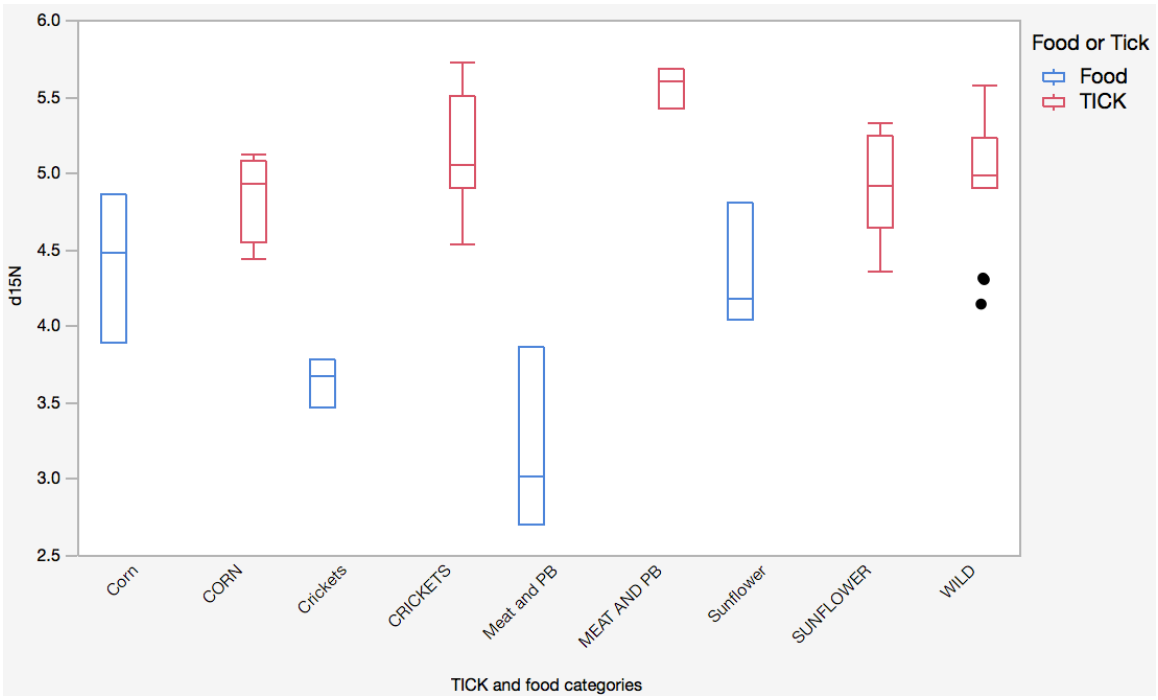

Figure 4. Outlier box plot comparing each food group and each group of ticks. Ticks: meat (n=3), insect (n=6), wild (n=15), Neutral (n=6), corn (n=4). Food: CORN, CRICKETS, MEAT and PB, NUT, and WILD (n=3 each).

Discussion:

This experiment was designed to explore the potential effects of different host diets on the isotopic signatures of ticks. Using prior studies and data, the presumed results seemed to be apparent. The corn registered the highest $\delta^{13}C$ of all the food groups, which was expected based on the results of LoGiudice et al. (2018), and studies from Kelly (2000) and DeMots (2010). The neutral and insect diets were found to be in a reasonable range within the extremes of the meat and corn diets. The corn ticks were expected to have the highest $\delta^{13}C$ levels reflecting the high $\delta^{13}C$ signature of the corn their chipmunk hosts fed on. LoGiudice et al. demonstrated this connection. The data based on 16 corn-fed animals showed ticks having $\delta^{13}C$ ranging from -20.6 to -23.9‰, expectedly providing highly enriched $\delta^{13}C$ signatures.

However, the corn ticks in this study have the lowest $\delta^{13}C$ of all the dietary groups (about -25.5 to -24.5 ‰), showing that the $\delta^{13}C$-enriched diets of the chipmunks were not reflected in the ticks that fed on them as demonstrated by LoGiuidice et al. This is perplexing and needs further investigation. It may be due to the higher water content of the canned corn provided to the chipmunks (LoGiudice et al. used dried and frozen corn). Thus, the animals may have consumed very little corn, rather, they may have consumed mostly the water in the corn, resulting in an unexpectedly low $\delta^{13}C$ that does not resemble a signature typical of corn. The very low sample size (n=4 ticks from 3 animals) should also be noted.

It was expected that the $\delta^{15}N$ would be enriched from food to tick, since the signature would span two trophic levels and thus, undergo dramatic trophic enrichment. The signatures observed generally did undergo enrichment, however not enrichment dramatic enough to span two trophic levels. When the meat (average $\delta^{15}N$ = 3.5‰) was compared to the meat ticks, their signatures contrasted the respective diet. Meat ticks showed the highest $\delta^{15}N$ signatures of all the tick groups while the meat diet ticks showed surprisingly low values (average $\delta^{15}N$ = 5.5‰). Since the ticks were two trophic levels removed from the meat, it was expected that their signatures would be enriched by 4‰ to 8 ‰, but were only enriched 2‰. The same pattern was found in all other food groups and their corresponding ticks. Due to the small sample size not much can be determined from this. Franta (2012) shows the primary nutrient in a tick's diet is hemoglobin, however the hemoglobin turnover rate is far too slow to be a determining factor in stable isotopic signature in a short-term study such as this. MacAvory (2006)

studied the half-life of red blood cells in rats and mice, finding the isotopic signatures of red blood cells in regards to carbon had a range of 24.8 days and 17.3 for half-life, while nitrogen had 27.7 and 15.4 days. Since chipmunks are a rough intermediate size between the two, there is reason to believe that their red blood cell turnover rate would be in that range. This implies that new nutrients being absorbed through diet would not incorporate significantly into red blood cells in a short time period since they take a long period of time to turnover or replace old red blood cells with new ones. Initially this short time frame was used under the assumption that, similar to the carbon signatures, the nitrogen signatures would change rapidly and that they would be incorporated into the tick in a short period of time. It is now clear that there may be no simple nitrogen-containing molecule that is rapidly incorporated into the tick.

Conducting additional studies would be particularly useful in determining whether or not nitrogen has utility in differentiating what host organism a tick has fed on. Stable isotope analysis should be used, specifically, to look into the timeframe of nitrogen stable isotope signatures in ticks and how drastically these signatures vary based on the host organism the tick has fed on. This study and previous work in the LoGiudice lab reveal that nitrogen isotopes may have a slower turnover rate and may be less sensitive than carbon isotopes to variation in host diets. Data from Scott (2015) compared the isotopic signatures of field collected ticks and those that had been fed on lab mice showed $\delta^{15}N$ signatures with a narrow range between roughly 4 to 9 per mil while $\delta^{13}C$ signatures ranged from -26 to -22 per mil. Similarly in my study, the range of $\delta^{15}N$ of ticks in the same diet groups was

much smaller than that of $\delta^{13}C$. Although the value ranges for $\delta^{15}N$ and $\delta^{13}C$ are rather similar, it does not mean the changes in $\delta^{15}N$, like $\delta^{13}C$ were dramatic. If $\delta^{15}N$ changed as expected, its range would be far greater than that of $\delta^{13}C$ due to trophic enrichment. These data suggest that small variations to diet in a short time frame may not impact nitrogen signatures as significantly as carbon signatures, which are known to have a quick turnover rate.

The results of this study point to flaws in the experimental methods. The first issue to be addressed is the fickle nature of administering very particular diets to wild animals and how to implement them in a way that delivers consistent and easily observable data and results. Preparing homogenized diets was an effort to achieve this, however it proved to be unpalatable to the hosts. In order to appropriately assess the impact of host diets on tick isotopic signature, extreme care must be applied to supplying diets that provide distinct isotopic signatures but are homogenized. Limiting hosts' access to supplementary food is also imperative. Additional food sources only complicate results and make clear dietary choices difficult to discern.

A second problem was the length of the captive period. It is also crucial that hosts are kept for a longer period of time than the four-day schedule implemented in this study. Often ticks that had fallen off chipmunks could not be discerned as ticks that had been naturally found on the host from the wild or ticks that we had placed on the chipmunks. This issue prevented me from precisely coordinating feeding schedule of the tick and its host. It is crucial to sync the tick feedings with

the host containment (giving adequate time for ticks from the field to fall off and placing ticks on hosts when or after their feeding regiment begins).

This study additionally revealed, the need to address the physiology of tick feeding and digestion. This aspect of ticks is paramount to understanding how isotopic signatures manifest and change depending on what ticks actually consume from their hosts and how it could translate to their signatures. What is currently known is that the primary nutrient hard-bodied ticks derive from their feeding is extracted from hemoglobin. The tick's digestive enzymes remove the heme and utilize the rest of the molecule for nutrients (Franta, 2012). The relatively quick incorporation of ^{13}C may be due to a tick's utilization of host glucose when feeding (LoGiudice et al., 2018). Beyond this not much is known about what other host fluids or compounds ticks utilize. This study addressed most controllable aspects of stable isotope testing on ticks and still did not produce definitive results. This suggests that a tick's unique digestive physiology is a big factor in establishing its isotopic signature and should be explored further in order to get a more comprehensive view on the stable isotope ecology of ticks.

Without this preliminary work it is difficult to approach and address all of the nuances of stable isotope analysis on ticks. This study reveals the significance of other details that were not accounted for, such as tick digestion, animal holding time and $\delta^{15}N$ characteristics. By demonstrating the sensitive and complex nature of stable isotope analysis in relation to ticks, this study emphasizes the need for further research testing these aspects of tick SIA.

Finally, these results demonstrated that nitrogen isotope might act differently than carbon isotope. Since carbon signatures are sensitive to small dietary shifts and take a relatively short amount of time to change, they are not very valuable for indicating what host a tick has fed on, since any small dietary change could significantly alter a signature. Nitrogen however, appears to be less sensitive. This study suggests that nitrogen isotopes are not influenced by short dietary changes and thus may be more useful in determining host use based on the tick's isotopic signature.

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